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Antisense Inhibitory Effect: A Comparison Between 3′-Partial and Full Phosphorothioate Antisense Oligonucleotides

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Abstract Phosphorothioate (PS) antisense oligonucleotides are currently used to inhibit many cell functions both in vivo and in vitro. However, these modified oligos provide reasonable sequence specificity only within a narrow concentration range. To overcome such a limitation we synthesized antisense oligomers, partially phosphorothioated, targeted against the human N-myc mRNA. We utilized such modified oligomers in a human neuroblastoma cell line where the N-myc gene expression was very high, and compared them to full phosphorothioate oligonucleotides. Both full PS and partial PS antisense oligos produced a maximum reduction in target mRNA after 6 h of treatment. They were able to maintain a good level of inhibition for 20 h only at high concentration. While partial PS oligos produced a dose dependent and sequence specific inhibition of N-myc mRNA, full PS molecules suffer from some disadvantages at the highest concentration used. Our results showed that partial PS molecules were capable of reducing gene expression showing a greater sequence specificity over a far broader concentration range. For this reason we conclude that partial PS antisense oligos, with respect to full PS antisense oligos, might be particularly useful for studying gene function. J. Cell. Biochem. 74:31–37, 1999.

Key words: antisense oligonucleotides; chimeric oligonucleotides; N-myc; neuroblastoma

Synthetic antisense oligonucleotides have been used successfully to inhibit many cell function both in vivo and in vitro [Crooke, 1992; Stein and Cheng, 1993; Dean and McKay, 1994; Hélène, 1994; Chadwick and Cardew, 1997; Romano, 1998]. Oligonucleotide activity depends upon many factors, including affinity towards the target mRNA, cellular uptake, and nuclease stability [Agrawal, 1996; Chadwick and Cardew, 1997; Stein and Krieg, 1998].

Phosphorodiester oligonucleotides were the first molecules used in antisense researches, but they were found to be rapidly degraded in biological systems [Chadwick and Cardew, 1997; Stein and Krieg, 1998]. To overcome this limitation, a number of other structural types of oligos with modifications of the phosphate backbone have been tested. Phosphorothioate oligonucleotides (PS), with one of the oxygens in the phosphate backbone replaced by sulfur, have became common as reagents because of their improved resistance to enzymatic degradation [Hélène and Saison-Behomaras, 1994; Stein and Krieg, 1998]. The nuclease stability of PS oligos allows effective oligonucleotide-dependent in vivo inhibition gene expression. However, PS oligos, in contrast to nonmodified oligos, provide reasonable sequence specificity only within a narrow concentration range [Summerton et al., 1997]. In fact, at high concentration they generate several "nonantisense" effects, such as inhibition of cell-matrix interaction and antiproliferative activity. These "non antisense" effects may result, at least in part, from the ability of PS molecules to bind cellular proteins [Guvakova et al., 1995; Agrawal, 1996; Chadwick and Cardew, 1997].

Abbreviations used: FCS, fetal calf serum; HPRT, hypoxanthine guanosine phosphoribosyl transferase; PS, phosphorothioate oligonucleotides.

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Fifteen mer chimeric antisense oligomers against human N-myc mRNA have been synthesized, with 11 normal phosphodiester internucleoside linkages and three phosphorothioate linkages at 5′ and 3′ termini, respectively. The 3′ terminal phosphorothioate section should protect the oligos from degradation by the most active serum and cellular nucleases, and may eliminate inappropriate activity which is attributed to full thioate oligos.

In order to verify the effectiveness of such chimeric oligos under extreme conditions, i.e., when the target gene is expressed at high level. We utilized 3′ partially thioathed oligomers in a human neuroblastoma cell line with a very high expression of N-myc gene [Foley et al., 1991]. In neuroblastoma tumor cells, a high level of N-myc expression has been correlated with advanced disease and rapid disease progression [Foley et al., 1991]. Full phosphorothioate oligonucleotides were used to provide a control comparison with the chimeric oligomers. Both "nude" and encapsulated oligos were tested in cell cultures supplemented either with untreated serum or with heat inactivated serum.

MATERIALS AND METHODS

Cell Cultures

SKNBE(2)C human neuroblastoma cells, grown in monolayer, were maintained at 37°C, 5% carbon dioxide in RPMI containing either 15% fetal calf serum or 15% heat inactivated fetal calf serum.

Synthesis of Oligodeoxyribonucletides

Synthesis of oligodeoxyribonucleotides was carried out on automatic DNA synthesizer (Model 200A, Beckman Instruments, Fullerton, CA) using the beta-cyanoethyl phosphoroamidite chemistry, as already reported [Iacomino et al., 1994]. The phosphorothioate oligonucleotides utilized to regulate N-Myc gene expression were as follow: ANTISENSE 5′-GAT CAT GCC CGG CAT, complementary to the mRNA region encompassing AUG starting codon [Ibson and Rabbitts, 1988]; REVERSE 5′-TAC GGC CCG TAC TAG; SCRAMBLED 5′-GAG CAT TCC CAG CGT. The phosphodiester oligonucleotides for RT-PCR were as follows: N-MYC1382 5′-AGG ACA CCC TGA GCG ATT CAG, N-MYC1601 5′-GGA GAG GGG GCG GCA TAG TTG for the N-myc cDNA; H3 5′-CCT GCT GGA TTA CAT TAA AGC ACT G, H8 5′-CCT GAA GTA CTC ATT ATA GTC AAG G for the HPRT cDNA [Galderisi et al., 1996].

Proliferation Assay

Neuroblastoma cells were seeded at 15,000/flask in 2 ml of medium. N-myc antisense, reverse or scrambled oligos were added at a final concentration of 10 and 20 μM, after 24 h. Cells then were harvested after another 24 h and the viable cells were counted in a hemocytometer by trypan blue exclusion.

Liposomes Treatment of Cell Cultures

Oligonucleotide molecules were incubated with DOTAP reagent (a liposome formulation of cationic lipids from Boehringer-Mannheim Biochemicals, Indianapolis, IN) and added to cell cultures.

RNA Extraction and RT-PCR

Total RNA was extracted from cell cultures using RNAzol reagent (Biotecx Laboratories Inc., Houston, TX) according to the manufacturer protocol of. Levels of N-myc mRNA were measured by RT-PCR amplification [Galderisi et al., 1996]. As controls appropriate regions of the hypoxanthine guanosine phosphoribosyl transferase (HPRT) cDNA were amplified. Amplifications were carried out for 28–30 cycles, using the following conditions: 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min. Each RT-PCR experiment was repeated at least three times. Products of amplifications were electrophoresed on 2% agarose gel in 1×TBE. Semiquantitative analysis of mRNA levels was carried out by "GEL DOC 1000 UV FLUORESCENT GEL DOCUMENTATION SYSTEM" (Bio-Rad, Richmond, CA).

RESULTS

In initial experiments "nude" oligomers were added at final concentrations of 1 μm and 5 μm to culture neuroblastoma cells growing in a medium containing heat inactivated serum. Because several authors have evidenced problems in interpretation of data derived from in vitro and in vivo use of antisense oligonucleotides containing stretches of dG and dC in their sequences [Yaswen et al., 1993; Chadwick and Cardew, 1997; Stein and Krieg, 1998], to claim a specific antisense effect of our molecules we used two control oligos containing dG/dC stretches, as they appear in the antisense se-

quence. These control oligos should be more suitable than sense oligos to investigate specificity of target mRNA inhibition. N-myc mRNA levels in cultured cells treated with thioated oligos were determined after normalization with respect to hypoxanthine phosphoribosyl transferase mRNAs, chosen as internal control.

Initially, the effects of oligos on target mRNA were monitored after 6 h (Figs. 1, 2A,B). Full PS antisense oligonucleotides produced a clear reduction of N-myc mRNA compared to reverse and scrambled oligos; on the other hand, partial PS antisense molecules were less effective. The effects of the oligos were then evaluated after 20 and 48 h of treatment. The reduction of N-myc mRNA levels were low with both full PS and partial PS molecules (Fig. IIA,B; Table IA). To increase N-myc gene inhibition 10 µm and 20 µm antisense oligos were added at cell cultures. The reduction of targeted mRNA was evaluated after 6, 20, and 48 h (Fig. 2C,D; Table IA).

Both full PS and partial PS oligomers produced a remarkable reduction of N-myc mRNAs after 6 h but only the full PS molecules maintained a considerable level of inhibition at 20 h. Both the antisense molecules were ineffective at 48 hours (Table IA). However, the full PS molecules at 20 µm concentration showed a low nonspecific N-myc inhibition. In fact, the ratio of N-myc/HPRT levels was slightly reduced in cell cultures treated with reverse and scrambled oligonucleotides (Fig. 2D). To further investigate these "nonantisense" effects, we determined the proliferation rate of cell cultures which were treated either with full PS or partial PS molecules.

Full thioate antisense treated neuroblastoma cells showed a significant reduction of proliferation rate compared with untreated cells. However, also 20 µm reverse and scrambled full thioate molecules decreased the cell proliferation (Fig. 3A). Partial thioate antisense oligos determined a reduction of cell growth, while control oligos were completely ineffective (Fig. 3B).

Then we investigate the inhibition of cellular adhesion, which is another common "nonantisense" effect attributed to phosphorothioate oligos. The exposure of neuroblastoma cell cultures to 10 µm and 20 µm full and partial PS molecules did not show any detectable inhibition of cellular adhesion after 24 h of treatment (data not shown).

In order to improve inhibition of N-myc gene expression by antisense oligonucleotides, DOTAP cationic liposomes have been used to deliver oligomers into culture cells. DOTAP re-

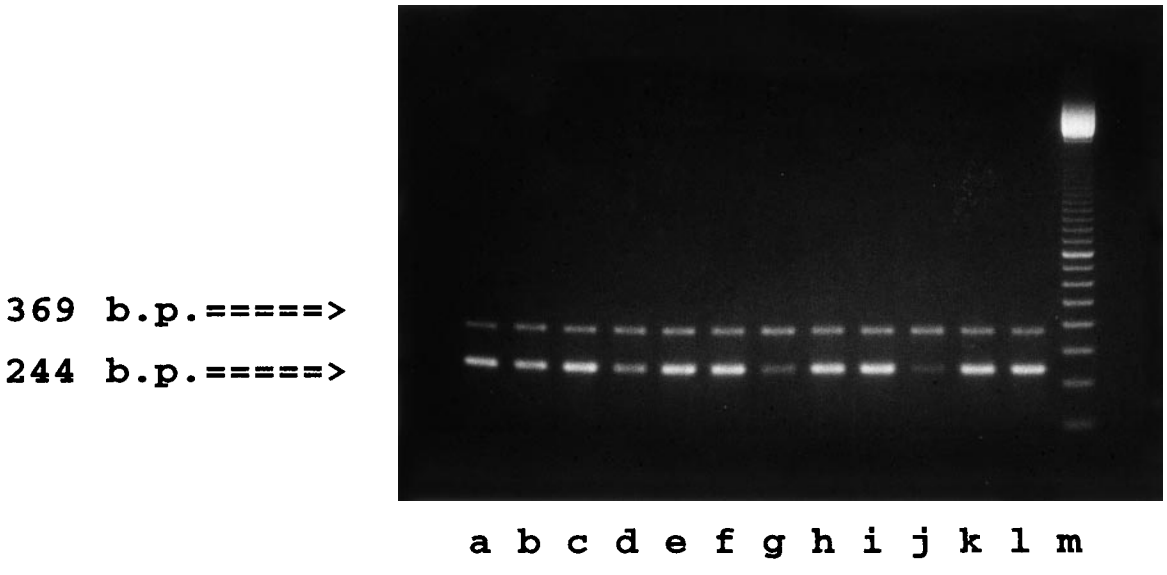

Fig. 1. Agarose gel electrophoresis analysis of RT-PCR products of N-myc and HPRT mRNAs. The relative position of the DNA fragments are indicated by arrows (369 b.p. for HPRT and 244 b.p. for N-myc, respectively). Cells were treated for 6 h with "nude" partial phosphorothioate oligos. **Lanes a,d,g,j:** Antisense oligos at 1 µm, 5 µm, 10 µm, and 20 µm concentration, respectively. **Lanes b,e,h,k:** Reverse oligos at 1 µm, 5 µm, 10 µm, and 20 µm concentration, respectively. **Lanes c,f,i,l:** Scrambled oligos at 1 µm, 5 µm, 10 µm, and 20 µm concentration, respectively. **Lane m:** 100 b.p. ladder as molecular weight marker.

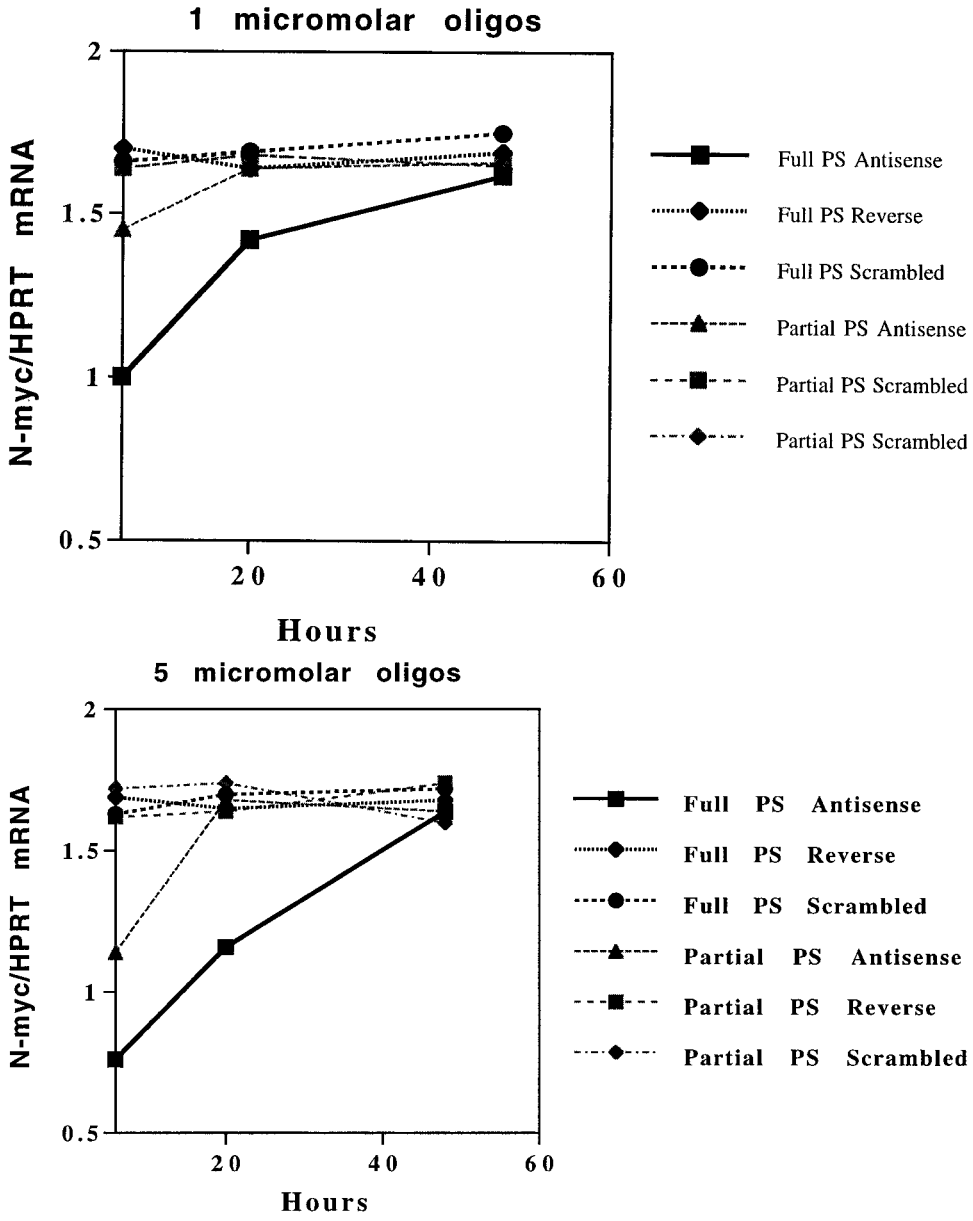

Fig. 2. N-Myc/HPRT mRNA ratios measured from neuroblastoma cell cultures treated with full PS and partial PS oligos as indicated in the inserts. Cells were treated with 1 µm (**A**), 5 µm (**B**), 10 µm (**C**), and 20 µm (**D**) oligos. The mRNA ratios was determined after 6 h, 20 h, and 48 h of treatment. Each value is the mean of three different experiments.

agent, not toxic for many cell lines, as already shown [Capaccioli et al., 1994] was combined with different concentrations (0.1, 0.5, 1, 5 µm) of oligomers. The effect on N-myc expression was determined after 6, 20, and 48 h and compared with "nude" oligo treatments (Table IA).

The reductions of N-myc mRNA levels after 6 h of treatment were slightly higher than those with both "nude" full PS and partial PS molecules. The maximum inhibition of gene expression was obtained with 0.5–1 µm of oligos. Increasing the oligos' concentration up to 5 µm did not improve the antisense effectiveness.

Interestingly, DOTAP-encapsulated antisense oligos showed a remarkable inhibition effect on N-myc expression after 20 h of treatment. The decrease of N-myc mRNA was significantly higher than that obtained with "nude" molecules and it was still observed after 48 h of treatment when "nude" molecules were ineffective. Even in these conditions the partial PS antisense molecules were less effective than the

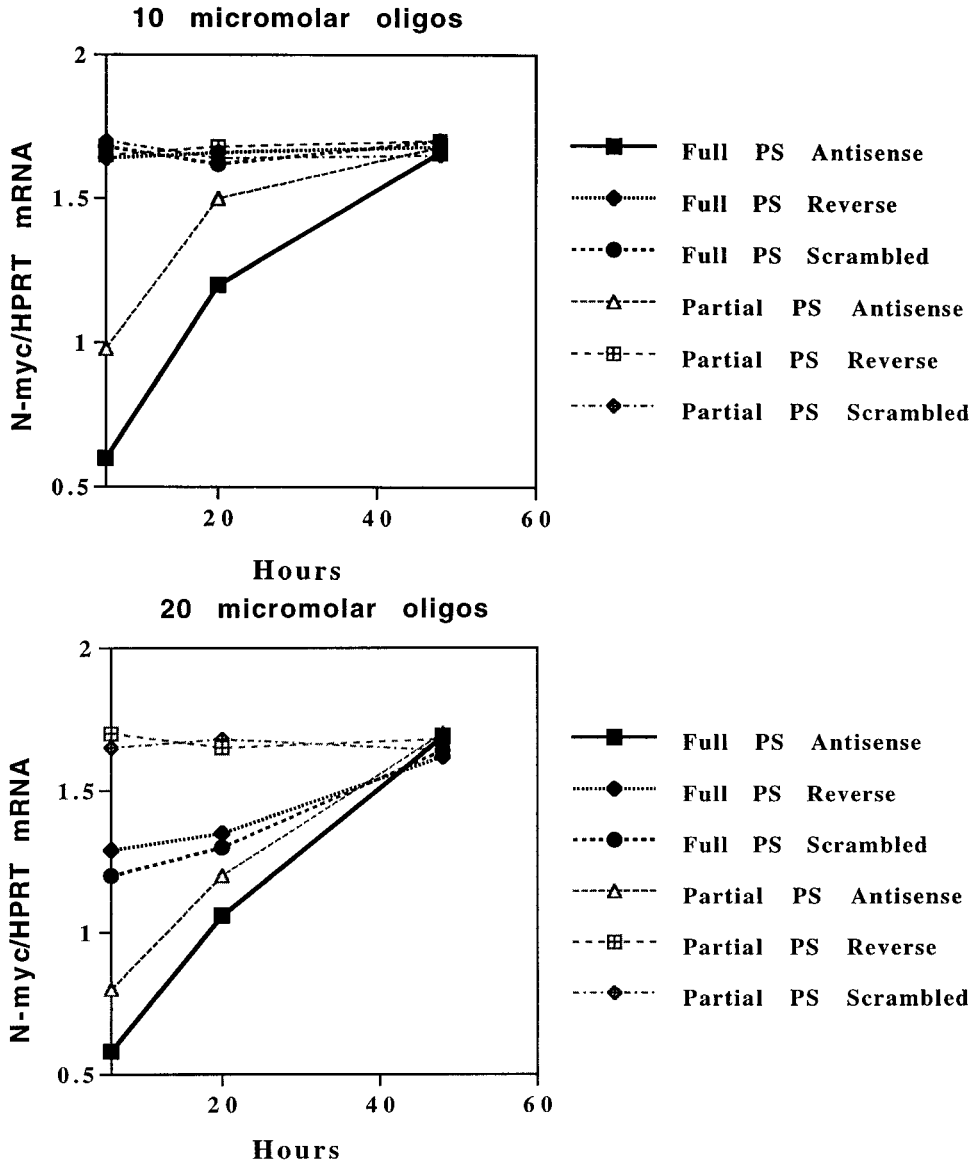

Figure 2. (Continued.)

full PS oligos. However, these latter molecules showed a slight nonspecific N-myc inhibition at 5 μm concentration. Serum added to cell culture medium contains active nucleases mostly 3′ exonucleases, that can degrade oligonucleotides.

To determine the influence of serum nucleases on the effectiveness of antisense oligos we performed some experiments adding oligonucleotides to cell cultures containing normal fetal calf serum. We utilized the oligos' concentrations which gave the best results in cell cultures supplemented with heat inactivated serum. Full PS oligos and partial PS molecules were added to neuroblastoma cultures at 10 μm concentration as "nude molecules" and at 1 μm as DOTAP encapsulated. Results were evaluated after 6, 20, and 48 h of treatment (Table IB).

The inhibition of N-myc expression was lower than that observed in medium containing heat inactivated serum. For example, after 6 h of treatment "nude" full PS produced a 38% reduction in N-myc mRNA compared to 63% reduction observed in medium supplemented with heat inactivated serum (Table IA,B). Furthermore, "nude" partial PS antisense oligomers were greatly influenced by active serum nucleases. DOTAP liposomes partially protect oligomers from nuclease degradation (see Table I).

TABLE I. N-myc mRNA Level Reduction in Antisense Treated Cells Compared to Control Oligo Treated Cells[a]

Nude oligomers[b]

	Full PS			Partial PS		
	6 h	20 h	48 h	6 h	20 h	48 h
1 μM	40 ± 3	13 ± 1	ND	21 ± 2	ND	ND
5 μM	55 ± 6	30 ± 4	ND	31 ± 4	ND	ND
10 μM	63 ± 5	38 ± 5	ND	40 ± 3	20 ± 2	ND
20 μM	53 ± 4#	20 ± 3#	ND	48 ± 4	19 ± 2	ND

Encapsulated oligomers

	Full PS			Partial PS		
	6 h	20 h	48 h	6 h	20 h	48 h
0.1 μM	36 ± 3	30 ± 3	ND	28 ± 4	19 ± 2	ND
0.5 μM	60 ± 6	49 ± 3	30 ± 2	55 ± 4	40 ± 3	20 ± 3
1 μM	68 ± 6	50 ± 4	35 ± 3	60 ± 4	40 ± 2	23 ± 3
5 μM	49 ± 4#	34 ± 3#	19 ± 3	62 ± 5	39 ± 4	21 ± 2

	6 h	20 h	48 h
10 μM Nude full PS[c]	38 ± 2	18 ± 2	ND
10 μM Partial ps	15 ± 2	19 ± 3	ND
1 μM Encapsulated full ps	46 ± 4	30 ± 3	ND
1 μM Encapsulated partial ps	18 ± 4	18 ± 2	ND

[a]The mRNA reduction was calculated as follows: [1-[2A/(R + S)]] × 100, where A, R, and S are the ratios of N-myc/HPRT mRNA levels in antisense, reverse, and scrambled treated cells, respectively. Each determination was the mean of at least three different experiments. ND, not detectable. #, the percentage of N-myc reduction could be underestimated (see Results).
[b]Neuroblastoma cells grown in medium supplemented with heat inactivated FCS.
[c]Neuroblastoma cells grown in medium supplemented with normal FCS.

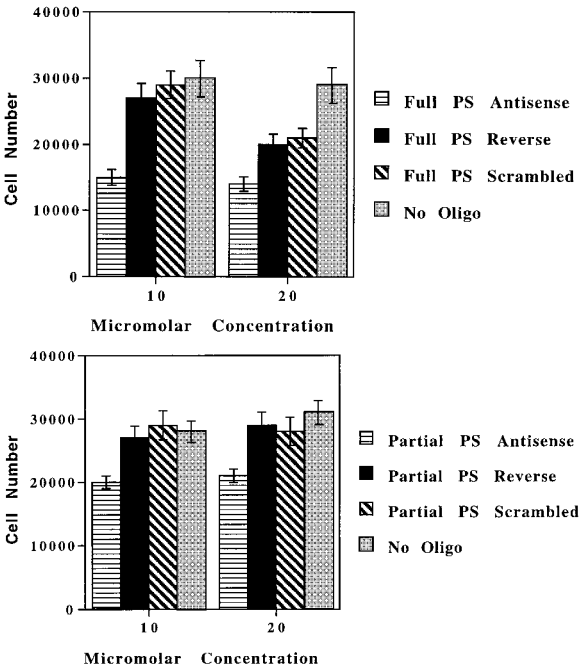

Fig. 3. A,B: Neuroblastoma cells were seeded at 15,000/flask in 2 ml of medium. After 24 h N-myc antisense, reverse or scrambled oligos were added to the final concentrations as indicated in the graphics. After further 24 h of incubation, cells were harvested and the number of viable cells was determined.

DISCUSSION

The identification of a 15mer oligodeoxynucleotide which reduces human N-myc expression in neuroblastoma cell lines has been previously described [Rosolen et al., 1990; Negroni et al., 1991]. However, these experiments suffer from some drawbacks: the authors utilized a high concentration of normal phosphodiester antisense oligonucleotides and only sense oligonucleotides as control.

We used full PS and partial PS antisense oligos to obtain molecules effective at low concentration and with increased lifespan, performing also experiments on neuroblastoma cells cultured in medium containing active serum nucleases to check the true resistance of oligos to degradation.

The kinetics for reduction in N-myc mRNA were determined after a single treatment with antisense oligos supplemented at different concentration to neuroblastoma cell cultures by direct addition. Both full PS and partial PS antisense oligos produced a maximum reduction in target mRNA after 6 h of treatment. They maintained a good level of inhibition till

to 20 h only at high concentration. Control oligos were totally ineffective. Full PS oligos at 20 µm concentration showed a slight non specific N-myc inhibition. This was confirmed by the proliferation rate reduction observed in cultures which were treated either with reverse or scrambled full PS oligos at 20 µm concentration.

All these findings indicate that partial PS oligos produced a dose-dependent and sequence-specific inhibition of N-myc mRNA, while full PS molecules suffer from some disadvantages at our highest concentration.

We then used cationic lipids for a functional delivery of oligos in cultured cells. Generally, we observed only a slight enhancement of N-myc gene inhibition. However, the inhibition was obtained with a lower oligo concentration. Furthermore, we noticed a prolonged antisense effect.

Thus, it should pointed out that DOTAP liposomes are effective mainly in delaying oligo degradation. The oligos' effect was subsequently analyzed in neuroblastoma cells cultured in medium supplemented with normal FCS. The effectiveness of antisense partial PS was greatly reduced while full PS molecules were still active.

In conclusion, our results showed that partial PS molecules were capable of reducing N-myc gene expression in cell cultures and in comparison with full PS molecules, they afforded greater sequence specificity over a far broader concentration range. For these reasons such oligos in combination with liposome reagent may be particularly useful for studying gene function, when a very specific inhibition of the target gene is necessary for a proper understanding of its role in the cell. However their use should be limited to cell cultures growing in a medium supplemented with heat inactivated serum.

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REFERENCES

Agrawal S. 1996. Antisense therapeutics. Totowa, NJ: Humana Press.

Capaccioli S, Di Pasquale G, Mini E, Mazzei T, Quattrone A. 1993. Cationic lipids improve antisense oligonucleotide uptake and prevent degradation in cultured cells and in human serum. Biochem Biophys Res Comm 197(2): 818–825.

Chadwick DJ, Cardew G. 1997. Oligonucleotides as therapeutic agents. West Sussex, UK: John Wiley & Sons.

Crooke ST. 1992. Therapeutic application of oligonucleotides. Annu Rev Pharmacol Toxicol 32:29–376.

Dean NM, McKay R. 1994. Inhibition of protein kinase C-alpha expression in mice after systemic administration of phosphorothioate antisense oligodeoxynucleotides. Proc Natl Acad Sci USA 91:11762–11766.

Foley J, Cohn SL, Salwen HR, Chagnovich D, Cowan J, Mason KL, Parysek LM. 1991. Differential expression of N-myc in phenotypically distinct subclones of a human neuroblastoma cell line. Cancer Res 51:6338–6345.

Galderisi U, Cipollaro M, Melone MAB, Iacomino G, Di Bernardo G, Galano G, Zappia V, Cascino A. 1996. Myotonic dystrophy: Antisense oligonucleotide inhibition of DMPK gene expression in vitro. Biochem Biophys Res Comm 221:750–754.

Guvakova MA, Yakubo LA, Vlodavsky I, Tonkinson JH, Stein CA. 1995. Phosphorothioate oligodeoxynucleotides bind to basic fibroblast growth factor, inhibits its binding sites on extracellular matrix. J Biol Chem 270(6):2620–2627.

Helene C. 1994. Rational design of sequence-specific oncogene inhibitors based on antisense and antigene oligonucleotides. Eur J Cancer 30A:1721–1726.

Helene C, Saison-Behomaras E. 1994. la stratègie antisense: Nouvelles approches thèraupeutiques. Medicine/ Science 3(10):253–273.

Iacomino G, Galderisi U, Cipollaro M, Di Biase S, La Colla P, Marongiu ME, De Rienzo A, De Falco S, Galano G, Cascino A. 1994. Phosphorothioated antisense oligonucleotides: prospects for AIDS therapy. Life Science Advances: Mol Biol 13:69–74.

Ibson JM, Rabbitts PH. 1988. Sequence of a germ-line N-myc gene and amplification as a mechanism of activation. Oncogene 2(4):399–402.

Negroni A, Scarpa S, Romeo A, Ferrari S, Modesti A, Raschellà G. 1991. Decrease of proliferation rate and induction of differentiation by MYCN antisense DNA oligomer in a human neuroblastoma cell line. Cell Growth Diff 2:511–518.

Romano G, Claudio PP, Kaiser HE, Giordano A. 1998. Recent advances, prospects and problems in designing new strategies for oligonucleotides and gene delivery in therapy. In Vivo 12:59–68.

Rosolen A, Whitesell L, Ikegaki N, Kennett RH, Neckers LM. 1990. Antisense inhibition of a single copy N-myc expression results in decreased cell growth without reduction of c-myc protein in a neuroepithelioma cell line. Cancer Res 50:6316–6322.

Stein CA, Cheng YC. 1993. Antisense oligonucleotides as therapeutic agents is the bullet really magical. Science 261:1004–1012.

Stein CA, Krieg M. 1998. Applied antisense oligonucleotide technology. Springer-Verlag: New York, NY.

Summerton J, Stein D, Ben Huang S, Matthew,Weller D, Partridge M. 1997. Morpholino and phosphorothioate antisense oligomers compared in cell-free and in cell system. Antisense Nucl Acid Drug Dev 7(2):63–70.

Yaswen P, Stampfer MR, Ghosh K, Cohen JS. 1993. Effects of sequence of thioated oligonucleotides on cultured human mammary epithelial cells. Antisense Res Dev 3:67–77.